FORMATION OF ENDODERM FROM ECTODERM IN CORDYLOPHORA

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There has been no satisfactory resolution for the problem of the extent to which one type of differential animal cell may be converted to that of another type. In one guise or another this question has come into prominence a number of times during the past half century. Several decades ago the issue was whether "dedifferentiation" followed by "redifferentiation" in a new direction is possible. The observations of H. V. Wilson (1907, 1911a, 1911b) on reconstitution from dissociated sponges and hydroids stimulated one phase of interest in the problem. Contributions to the discussion were made by many outstanding biologists of that time (*e.g.,* J. S. Huxley, C. M. Child, J. Loeb, T. H. Morgan, etc.). Later, experiments with cultured tissues led to the general observation that rapidly proliferating tissues lose their characteristic morphological appearance. This stimulated more research and thinking on the "dedifferentiation-redifferentiation" issue. The major aspects of the current attitudes in this field have been reviewed by Trinkaus (1956) and Grobstein (1959).

In general there are two schools of thought: one, which maintains that cell types are irreversibly fixed and that one type of cell cannot be transformed to another (except within a rather narrow "modulatory" range) and the other, which claims that at least some types of cell transformation of a more radical sort (metaplasia) are possible. A number of well substantiated cases of metaplasia have been described in recent years. Our knowledge of Wolffian regeneration (formation of a lens from the iris of the eye) has been expanded by L. S. Stone and collaborators (1952). Fell and Mellanby (1953) and Weiss and James (1955) have demonstrated that the ordinarily squamous epithelium from the skin of 6-day chick embryos may be converted to a mucous secreting columnar epithelium by exposure to excess vitamin A. The tissues of the chorio-allantoic membrane of chick embryos may change in response to a variety of conditions (Moscona, 1959, 1960; Moscona and Carneckas, 1959). Other cases may be cited. There also are well substantiated cases which indicate that some tissues and some cell types are quite stable and do not transform to other types. This is especially true of so-called terminal tissues, *i.e.,* tissues which have achieved an extreme state of functional maturity (possibly involving eventual cell sloughing) or extreme cell specialization. Ordinarily such cells do not divide. There remains a broad area in which the picture is not clear and it seems as though the best approach to the problem is to state it in some form as "which cell types in which tissues are stable and which are sufficiently unstable so that they may undergo metaplasia under desig-

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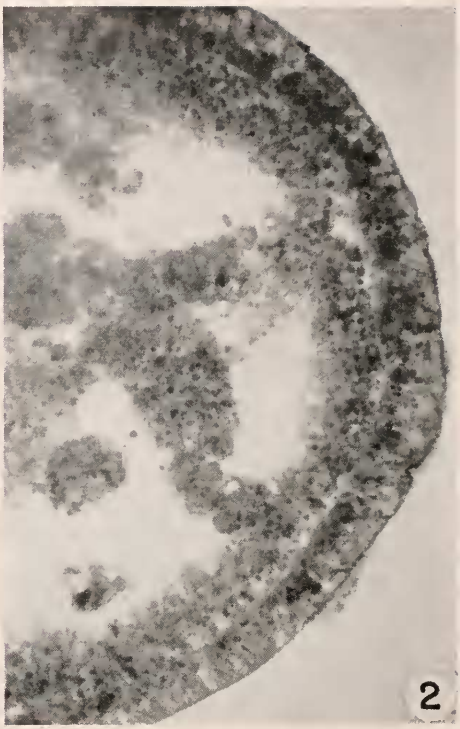
nated circumstances?" and then to design experiments which may answer this question.

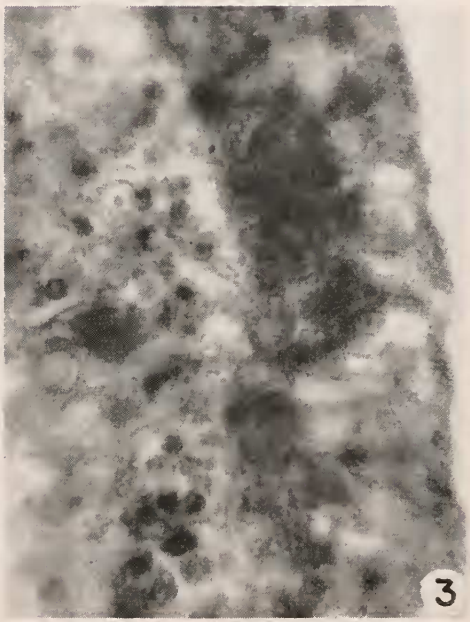
A complicating factor for this general problem is the possible existence, in many organisms and tissues, of reserve cells with "embryonic" capabilities. In some situations, as in the case of regeneration of amphibian limbs, the importance of reserve cells has been discounted (Butler, 1935). In other cases (*i.e.,* interstitial cells (I-cells) of hydroids, amebocytes of sponges and neoblasts of flat worms) the reserve cell notion still has wide acceptance (Brien and Reniers-Decoen, 1955; Tardent, 1960; Burnett, 1962). Because of this situation, answers to the questions raised above may be equivocal unless the experiments are designed to deal with progenies of single cells of known origin (*i.e.,* clones). Unfortunately, the technical aspects of cloning cells have not yet been perfected for most cells coming from a wide range of tissues and organisms. In fact, the application of cloning procedures is still quite restricted. Since this is the case our present approaches must be less direct and less decisive. This communication deals with an experiment of the latter sort. While not unequivocal, it makes a contribution to our understanding of cell capacities in hydroids.

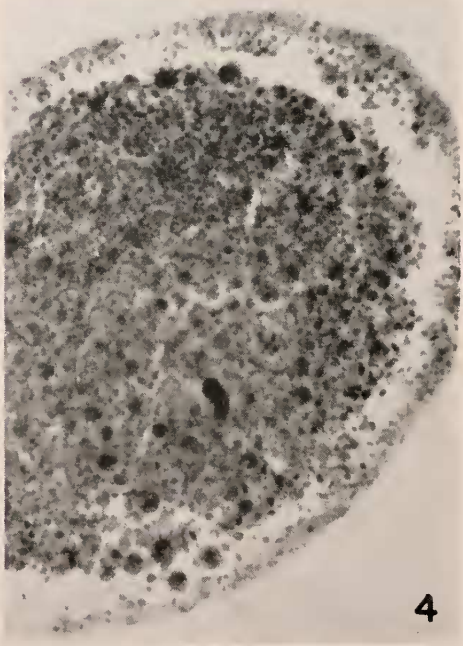
Three papers, dealing with reconstitution of hydroids, may be cited as typical of the attitudes one encounters in such studies. Gilchrist (1937) found that ectoderm and endoderm could be separated quite readily in scyphistomae of *Aurelia.* The mesoglea is sufficiently thick in these animals so that it may be cut with a knife, leaving ectoderm with one half and endoderm with the other. The endodermal sheets formed small ciliated balls which lived for days but did not reconstitute an individual. The ectoderm, on the other hand, did form an individual complete with an endodermal layer. Gilchrist felt that the new inner layer must have been formed by the interstitial cells.[2]

Beadle and Booth (1938) were, *inter alia,* concerned with the question of whether I-cells, largely restricted to ectoderm in *Cordylophora,* can form endodermal elements. To this end they separated ectoderm from endoderm in reconstitution masses made from the coenosarc of *Cordylophora.* Four masses of supposedly pure ectoderm were isolated. One of these developed a small hydranth and sections of two of the others revealed that they had begun to form an inner layer. The authors were uncertain of the nature of the inner layer in these two cases, since the cells were not typically endoderm. However, they felt that the fully reconstituted hydranth came from a case in which (p. 312) ". . . a small amount of endoderm must have been included. . . ." These are the two typical "explanations": I-cells formed the new tissue or some cells from the tissue in question "must have been included"! To complete the cycle I may cite Papenfuss and Bokenham (1939) who were unable to obtain reconstitution with either isolated ectoderm or endoderm from *Hydra.* These authors suggest the possibility that Gilchrist did not remove all of the endoderm from his supposed ectodermal isolates!

[2] Gilchrist did not include a histological study of his material with his account. Steinberg (1963) repeated Gilchrist's experiments with *Aurelia* scyphystomae and, from her histological study, found that these animals do not have typical interstitial cells. She did confirm the finding that a new endodermal layer formed from isolated ectoderm and presented evidence that proliferating ectodermal cells give rise to amoebocytic cells which then form a typical endodermal layer. The amoebocytes themselves do not divide actively; instead, the somatic ectodermal cells show considerable mitotic activity during the time that amoebocytes increase in number.







FIGURES 1–5.

Beadle and Booth dealt with only four masses of supposedly endoderm-free ectoderm, and an inner layer of some sort was formed in three of these. The original question which concerned Beadle and Booth was whether I-cells of the ectoderm can form endoderm. Their conclusion that an endodermal layer can form only when the ectoderm is contaminated with former endoderm cells and that I-cells in the ectodermal layer cannot form endodermal elements seems to be somewhat shaky in view of their scanty evidence. Before a judgment can be made on the chief problem about the developmental capacity of the ectodermal cells it is important to establish more firmly the answer to the first question: does an endodermal layer form only from pre-existing endodermal cells or, put the other way, can an endodermal layer form from uncontaminated ectoderm? This question has been subjected to close scrutiny with the *Cordylophora lacustris* found near Woods Hole as the test object. The results do not support Beadle and Booth but, instead, indicate that an individual, complete wth an endodermal layer, may be reconstituted from isolated ectoderm of *Cordylophora*.

Material and Methods

The *Cordylophora* used for these experiments was collected during July and August of two successive summers at the edge of a fresh-water pond. The best spot for collection was near a wooden run-off at one end of the pond. A small clump of the mature colony was placed in a jar with some of the pond water, taken to the laboratory and kept cool and aerated in the original pond water. Only fresh material (*i.e.*, in the laboratory no more than three days) was used in the experiments.

While the *Cordylophora* lived in fresh water, it was found that a more favorable medium for reconstitution of masses of coenosarc was 25–30% sea water (Beadle and Booth, 1938, used 50% sea water). The sea water was filtered, diluted with either tap or distilled water and then pasteurized. The pasteurized medium was cooled and aerated before use.

Healthy, relatively straight stems were selected. The distal hydranth was cut away, and the coenosarc was pushed out of the perisarc by means of a curved bit of thin glass rod or a bit of shaped Tygon tubing which was held in a pair of forceps. Usually the gleaming white ectoderm separated from the pigmented endoderm (yellow or orange) very readily (Fig. 1). An exposure of the stem to the 25% sea water for 5–30 minutes before the extrusion greatly facilitated the separation of layers.

Figure 1. Newly extruded coenosarc of *Cordylophora*. Note the separation of ectoderm and endoderm (darker tissue) in the upper portion of the preparation.

Figure 2. Section through 10-hour mass of equal parts of ectoderm and endoderm. Note the complete separation of the two layers and the presence of a thin mesogleal layer between the two. Note also that cells are intact.

Figure 3. High power view of same section as in Figure 2. Note the inclusions in the endothermal cells (at left).

Figure 4. Section through 10-hour mass of pure ectoderm. Note the thin outer wall and highly fragmented and disorganized contents of the hollowed sphere. Compare with Figure 2.

Figure 5. High power view of same section as in Figure 4. Note the character of the cells of the wall; also the interstitial cell adhering to its inner surface.

Beadle and Booth found that neutral red was taken up selectively by the endoderm and took advantage of this to distinguish the two layers. Since the natural pigmentation of the endoderm in our animals was so distinctive and the layers separated quite readily, no supplementary treatment was deemed necessary. Separation was completed with the aid of cataract knives and the ectoderm was removed to a clean dish for further inspection. The tissue was cut into small pieces (0.1–0.2 mm. in diameter) and each piece was carefully inspected, at high magnifications of the dissecting microscope (50–90 ×), for contaminating endoderm. Any pigmented material was removed from the ectoderm. Reflected and transmitted as well as oblique lighting were used to insure the identification and elimination of the orange endodermal cells. These could be seen most favorably against a white background. In this way there was reasonable certainty that all endodermal cells were removed. Several of the small masses were then heaped together and allowed to fuse and form a single mass. Masses were transferred to fresh medium in small Stender dishes and kept on a shaded part of the laboratory table.

Despite all of the precautions taken there was always the possibility that one or two endodermal cells could escape our scrutiny and be included inadvertently. In order to assess the consequences of this possibility we set up control masses of ectoderm to which minute traces (no more than 3–5 cells) of endoderm were deliberately added. In addition other controls with varying amounts of endoderm (up to 50%) were used.

The table-top temperature, which was recorded for each experiment, varied from 19° to 22° C. Ectodermal masses were fixed in Bouin's fixative at various times following isolation; these were embedded in paraffin and sectioned at 5–10 μ. Toluidine blue at pH 5 proved to be a favorable stain.

Results

A total of 117 viable masses of *Cordylophora* coensarc was prepared for this study. Forty of these consisted of pure ectoderm (*i.e.*, no endoderm discernible with high magnifications of the dissecting microscope), 40 had deliberately included traces of endoderm, and in the rest endoderm comprised up to 50% of the total tissue mass. Thirty-five masses were fixed at various times after isolation.

Pieces of extruded coenosarc fused readily and, in most cases, formed a smooth sphere. In favorable cases the sphere became hollow within five to 10 hours after isolation. Spheres which did not become hollow did not reconstitute, but remained in this inactive condition for days. The center gradually became opaque but the surface layer still retained the refractile appearance usually associated with living tissue. The hollowing-out process was noted by Beadle and Booth, who conjectured about the activity of ectoderm in transporting fluid to the interior of the mass.

Almost 50% of the "pure" ectoderm masses reconstituted an inner layer (as judged by the presence of clearly discernible flagellar motion in the enteron) and most of these (9) formed a complete hydranth in 5–9 days. The rest (4) remained as stolonic growths which persisted without change for a long time. Essentially the same behavior was noted in masses which had a minute trace of endoderm. In both of these classes of isolates many (circa 50%) of the masses remained in-

active (see above), and did not reconstitute. In sharp contrast all but one of the 50:50 masses reconstituted a hydranth (one formed a stolonic growth); none were inactive.

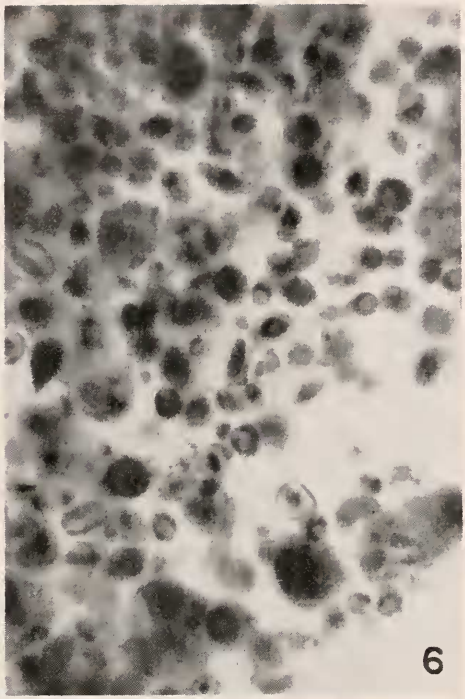
Most of the larger 50:50 masses formed a hydranth very quickly, within 30–36 hours. The average time for hydranth formation was 2–3 days. This is the average for all size classes; it was noted, however, that larger masses reconstituted a hydranth more quickly than smaller ones. When the masses are distributed into two groups, those larger and those smaller than 0.2 mm., the average time for hydranth formation for the group of larger isolates (6 masses) was 1.1 days and that for the smaller (13 masses) was 2.4 days. Moreover, the hydranths formed by most of the smaller mass were incomplete and had fewer tentacles than normal. A number of the large masses, on the other hand, developed more than one hydranth. (See Chalkley, 1945, for relation between initial mass and morphogenesis in *Hydra*.)

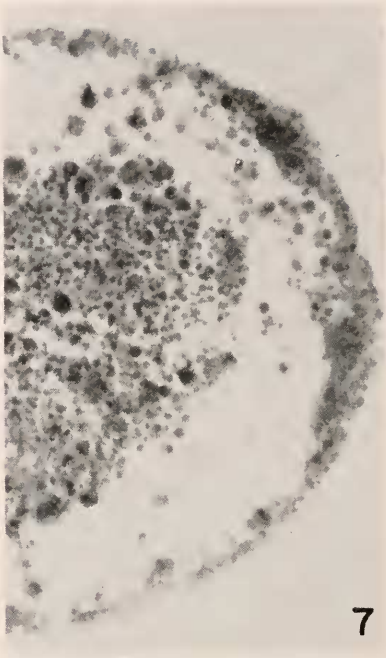
Histological Observations

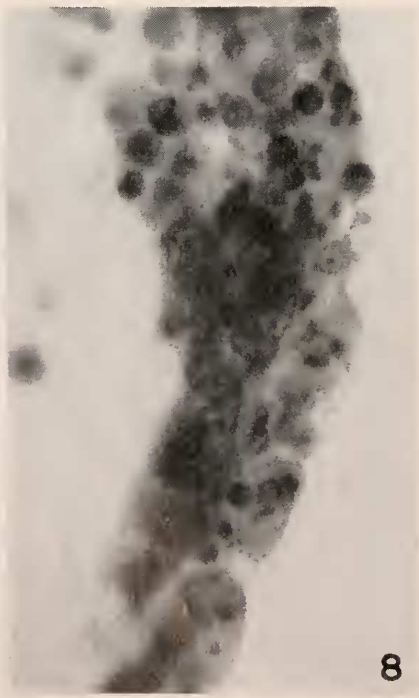
Our histological study was made on 25 masses (10 were lost or were not satisfactory for study) which were fixed at various times during reconstitution. Twelve of these were masses of supposedly pure ectoderm, eight were masses which had a trace of endoderm, and five consisted of equal amounts of ectoderm and endoderm.

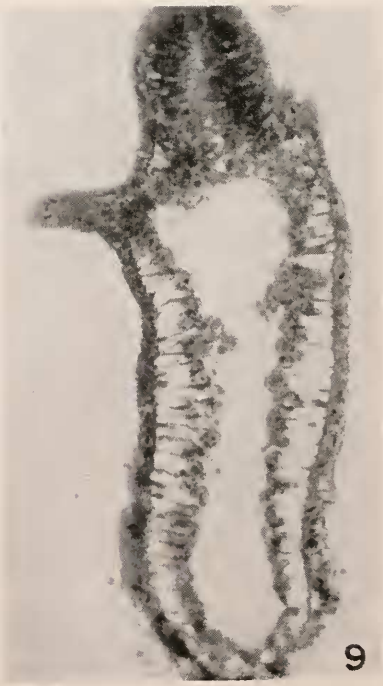
One of the first questions which required an answer was whether an occasional endoderm cell could be identified in the midst of a mass of ectoderm. Examination of sections of whole stems revealed that most of the endoderm cells were full of large inclusions of various sorts. This was the situation except in the hydranth and in the region immediately proximal to it where inclusion-free endoderm cells may be found. However, these regions were cut away before coenosarc was expressed and, therefore, most of the endoderm with which we are concerned was characterized by the presence of many inclusions. Ectoderm cells are not free of inclusion but rarely have more than one or two per cell. On the basis of this criterion we could ascertain that supposedly endoderm-free masses had no cells with more than 1–2 inclusions while a few cells with numerous inclusions could be identified in masses deliberately contaminated with traces of endoderm. The combination of this observation with the ease of identification of even single orange cells (endoderm) in the living mass when high magnifications of the dissection microscope were used provides reasonable confidence that our masses were endoderm-free. I doubt that one can make a stronger statement in a situation of this sort. Neither criterion is absolute; masses which are allowed to live can be, at the best, only good approximations of those sectioned.

The histological picture derived from masses of equal amounts of ectoderm and endoderm is quite clear and simple. The two layers rapidly sort out and, by the end of ten hours, form a definite inner endodermal layer which is separated from the ectoderm by a distinct basement-membrane-like mesoglea (Figs. 2 and 3). Cells of both layers are intact and retain the appearance which is typical of an intact stem. When the hydranth is elaborated many of the endoderm cells acquire the characteristics of typical secreting cells of the digestive portion of a hydranth. The two salient features are: cells remain intact, and sorting into two distinct layers is rapid.









FIGURES 6–9.

Since there was no observable difference between masses of "pure" ectoderm and those with traces of endoderm these two groups shall be treated together. Masses in these categories are characterized by a dramatic phenomenon. Sections of isolates which were fixed 1–2 hours after preparation revealed a picture which was quite similar to that seen in the ectoderm of sectioned intact stems. Somatic ectoderm cells and a number of I-cells could be distinguished clearly. By 8–10 hours all of the active masses were hollow and sections revealed that the relatively thin outer wall consisted of a single layer of small ectodermal cells. In isolates which were fixed at 10 hours or somewhat later, the contents of the sphere included, for the most part, cell fragments and scattered I-cells and relatively few recognizable intact somatic cells (Figs. 4, 5, 6). In some specimens it could be seen that a few I-cells were closely adherent to the inner surface of the cells of the sphere's wall (Fig. 5).

At this stage, it was evident that many small basophilic cells were scattered through the cell debris. Some of the somatic cell nuclei were pycnotic (this was confirmed, in another series, with iron hematoxylin-stained material) but there were not enough degenerating nuclei to account for the total number of initial somatic cells. On the other hand there was a marked increase in the number of small basophilic cells (I-cells) but no indication of mitotic activity.

During subsequent days the picture was essentially the same as for the 10-hour masses except that more I-cells accumulated on the inner surface of the wall (Figs. 7 and 8). By the fourth day a complete inner layer of enlarged cells was found in all masses which had shown signs of internal flagellar beating prior to fixation. Mitotic figures were not evident during the interval from 10 hours to 4 days. By the time a hydranth had formed (5–9 days), the inner layer was typically endodermal (Fig. 9). During the later phases of this process (4 days and following) the endodermal cells in many of the masses contained inclusions. The decrease in amount of cell debris in the hollow of the mass leads to the suspicion that the cells were phagocytizing the debris.

It is difficult to draw definite conclusions from static evidence of the sort presented above. There is the possibility that the picture reconstructed from this particular series is not typical. There is no certainty that the new endodermal layer forms from the interstitial cells which are seen adhering to the inner surface of the wall of the spheres. Surviving somatic ectodermal cells could, conceivably, make this inner layer. Efforts are under way to obtain more rigorous evidence which may shed light on these problems. However, one aspect of the original question seems reasonably clear: an endodermal layer does form from pure ectoderm. This point has been established more firmly by the following additional observations.

FIGURE 6. High power view (oil) of fragmented cellular material in interior of 24-hour ectodermal isolate. Note at least one small basophilic cell, many enucleated cell fragments and inclusions, and occasional (upper left) intact somatic cell. This photograph is from the same section as Figure 7.

FIGURE 7. Section of 24-hour ectodermal isolate. Note two groups of intensely basophilic cells adhering to the inner surface of the sphere's wall.

FIGURE 8. High power view of one of two groups of basophilic cells shown in Figure 7. Note the typical interstitial cell characteristics of these cells.

FIGURE 9. Section through a fully formed hydranth which has formed from an ectodermal isolate.

Two-Step Operation

Several *facts* could be derived from the observations described above. Within the first ten hours after separation of a mass of ectoderm a hollow sphere was formed, the wall of the sphere consisted of small ectodermal cells, all contaminating inadvertent or deliberately included endoderm cells were in the center of the sphere and were fragmented (or fragmenting) along with the rest of the ectoderm. In addition to this there were no I-cells *in* the ectodermal wall, but a few may have been adhering to its inner surface. An operation was carried out which could take advantage of the condition at 10 hours. Eleven ectodermal masses were prepared in the usual way. After 10–12 hours these were opened up and the inner contents were extruded and discarded. The inner surface of the walls was scraped clean of adherent material. The walls were then cut up and allowed to fuse together to form 5 second-generation reconstitution masses.

All of the second-generation masses became hollow and, by the sixth day after the second step of the operation, all five of them had an endodermal layer which could be detected by the definite flagellar movements which were readily discernible in the intact masses. Three of these elongated and formed stolonic growths while the other two formed typical tentacled hydranths by the end of the seventh day. This last experiment removes any doubt that an endodermal layer can form from isolated ectoderm of *Cordylophora*.

Discussion

The observations presented above indicate that the answer to the question of whether an endodermal layer can form from ectoderm of *Cordylophora* is different than that arrived at by Beadle and Booth. On the basis of a careful scrutiny of the material I feel confident that endoderm-free preparations of *Cordylophora* can reconstitute an endodermal layer. In addition to the histological confirmation of the absence of endoderm there is the two-stage operation which, in my mind, provides the most rigorous evidence in support of this contention.

Equally rigorous evidence about the ectodermal cell type which forms the endoderm is not at hand. Present observations do not allow us to ascertain whether only pre-existing interstitial cells or former somatic ectodermal cells contribute to the new layer. We hope to present additional evidence on this point in a subsequent publication. However, there is evidence from other forms which indicates that similar metaplastic changes may take place in the absence of I-cells. Normandin (1960) has reported (without details) that ectoderm-free endoderm of *Hydra* will reconstitute a new ectoderm when the endodermal isolate is free of I-cells. In my laboratory S. N. Steinberg (1963) has reinvestigated Gilchrist's observations with ectoderm from scyphistomae of *Aurelia*. She has confirmed the formation of an endodermal layer in the ectodermal isolates but her histological studies have failed to reveal an ectodermal cell type which has the characteristics of an I-cell. The nature of I-cells and their possible contribution to morphogenetic processes should, at the present, be regarded with a great many reservations.

The loss of tissue integrity and the accompanying cellular fragmentation which are typical of the ectodermal isolates of *Cordylophora* are striking phenomena. They appear to be very similar to the "histolysis" of muscle cells which has been noted in regenerating urodele limbs (Butler, 1935; Thornton, 1938; Hay, 1959)

where the muscle nuclei with some of the cell cytoplasm pinch off and acquire the characteristics of regeneration cells while the enucleated parts of the cell degenerate and are eventually removed by phagocytes. Cellular fragmentation of *Cordylophora* ectoderm seems to be related to an imbalance in the relative amounts of ectoderm and endoderm. In several cases in which single small masses (0.1 mm. in diameter) of endoderm were included with considerably larger amounts of ectoderm, tissue integrity was maintained locally, and a mesoglea was quickly re-established at the site of contact between endoderm and ectoderm. Ectoderm adjacent to this region of normal tissue association underwent the usual fragmentation. It is interesting to speculate that the key to the fragmentation may reside in whether or not a mesoglea persists or is re-formed. Isolated ectoderm of *Aurelia* scyphystomae does not undergo fragmentation; the thick mesoglea retains its usual relation to the cells. There seems to be a relationship between the persistence of a mesoglea or its rapid re-establishment and retention of cell integrity. This may merely be a coincidence, but it is an intriguing one.

Summary

Within ten hours after isolation of ectodermal masses of *Cordylophora lacustris*, a hollow sphere is formed in which the outer wall consists of a single layer of relatively small cells and most of the inner cells are fragmented and disorganized. During the course of 5–9 days an entire hydroid forms—complete with an endodermal layer. A two-stage operation, in which only the thin outer walls of 10-hour isolates were kept for further observation, also resulted in the formation of new endodermal layers. While it is not clear whether interstitial or somatic ectoderm cells are involved, it is evident that some ectodermal derivative forms the new layer. The possible relationship between cell fragmentation and absence of a mesoglea is noted.

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